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Quorum-sensing regulator RhlR but not its autoinducer RhlI enables *Pseudomonas* to evade opsonization

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Abstract

When *Drosophila melanogaster* feeds on *Pseudomonas aeruginosa*, some bacteria cross the intestinal barrier and eventually proliferate in the hemocoel. This process is limited by hemocytes through phagocytosis. *P. aeruginosa* requires the quorum-sensing regulator RhlR to elude the cellular immune response of the fly. RhlI synthesizes the autoinducer signal that activates RhlR. Here, we show that *rhlI* mutants are unexpectedly more virulent than *rhlR* mutants, both in fly and in nematode intestinal infection models, suggesting that RhlR has RhlI-independent functions. We also report that RhlR protects *P. aeruginosa* from opsonization mediated by the *Drosophila* thioester-containing protein 4 (Tep4). *RhlR* mutant bacteria show higher levels of *Tep4*-mediated opsonization, as compared to *rhlI* mutants, which prevents lethal bacteremia in the *Drosophila* hemocoel. In contrast, in a septic model of infection, in which bacteria are introduced directly into the hemocoel, *Tep4* mutant flies are more resistant to wild-type *P. aeruginosa*, but not to the *rhlR* mutant. Thus, depending on the infection route, the Tep4 opsonin can either be protective or detrimental to host defense.

Keywords competition opsonization-detection by pattern recognition receptors; infection route; intestinal infection; phagocytosis; quorum sensing
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Introduction

Opportunistic pathogens such as *Pseudomonas aeruginosa* or *Serratia marcescens* are able to infect a large range of hosts, from plants to humans [1,2]. They sense their environment and adapt their gene expression programs accordingly. Quorum-sensing (QS) systems allow pathogenic microorganisms to implement collective processes only when the pathogen number is locally high enough to ensure the efficiency of these processes, for instance, the expression of virulence factors or biofilm formation [3–5]. *P. aeruginosa* illustrates the complexity of QS signaling as its genome codes for at least three distinct intertwined QS systems that work in a hierarchical order (LasI/LasR > RhlI/RhlR > Quinolone Signal) [3,6–9]. The first two QS systems belong to the LuxI/LuxR category in which the LuxI enzyme synthesizes a homoserine lactone (HSL), respectively, 3-oxo-C12-HSL or C4-HSL, that activates its cognate receptor when a threshold concentration is reached [10–13].

Genetic model organisms such as the nematode *Caenorhabditis elegans* or the fruit fly *Drosophila melanogaster* have yielded unexpected insights into many key biological principles, including host–pathogen relationships [14–22]. Bacteria ingested either by worms or by flies are exposed in the gut lumen to antimicrobial peptides (AMPs) and reactive oxygen species produced by intestinal epithelial cells [23,24]. Whereas *P. aeruginosa* PA14 or *S. marcescens* remain mostly confined to the nematode gut lumen until late in the infection, a few bacteria cross the *Drosophila* intestinal barrier within 2 h. The bacteria do not initially proliferate and remain in low numbers in the insect hemocoel where they are controlled by phagocytosis [25–27]. Nevertheless, *P. aeruginosa* starts proliferating after a few days and systemic bacteremia ensues that ultimately kills the fly despite the induction of the systemic immune response that is mediated by the Toll and immune deficiency (IMD) pathways [28,29].

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The exact conditions of an intestinal infection modulate the outcome of infection and patterns of virulence. Thus, depending on the osmotic pressure in agar plates, nematodes can either succumb to phenazine-mediated "fast killing" or to a multifactorial "slow killing" [21]. Likewise, flies succumb to or survive ingested *P. aeruginosa* depending on the nature of the feeding solution [25]. The infection route also determines the outcome of an infection as the bacteria experience distinct environments within the host. Indeed, whereas intestinal infections with either *S. marcescens* or *P. aeruginosa* kill flies slowly, the introduction of as few as 10 bacterial cells through a septic injury directly in the hemocoel kills flies within 24 and 36 h, respectively [25–27]. Bacteria injected directly into the hemocoel immediately proliferate exponentially despite the release in the hemolymph of AMPs synthesized in the fat body [28,29].

Flies immunodeficient either for the cellular immune response or for the Toll or IMD pathways succumb sooner to an intestinal infection than wild type [25]. Previously, we found that among the *P. aeruginosa* PA14 QS systems, only RhlR is required for virulence of ingested bacteria that have escaped into the hemocoel, as it allows them to elude the cellular immune response by an unknown mechanism [25]. This infection model thus underscores the importance of the cellular arm of host defense against Gram-negative bacteria, which is overshadowed by the IMD systemic immune response in septic injury models [30–32]. One of the major functions of cellular immunity in adult flies is the phagocytosis of microbes present within the hemocoel. In vertebrates, phagocytosis is enhanced by the prior complement-mediated opsonization of microbes. Analysis of the *Drosophila* genome revealed the existence of complement family/α2-macroglobulin homologs known as thioester-containing proteins (TEPs). Most TEPs encode a GCGEQ motif in which an unstable bond between cysteine and glutamic acid has the potential to bind to hydroxyl or amine groups present on biological surfaces [33,34]. TEPs are found in many protostome groups and have been most intensively studied in mosquitoes and *Drosophila* [33,35–42]. In the mosquito *Anopheles gambiae*, Tep1 plays a key role against infection by *Plasmodium* malarial parasites [35,43].

The *Drosophila* genome encodes five functional *Tep* loci and a pseudogene (*Tep5*) [33]. The expression patterns of *Tep1-4* have been documented by *in situ* hybridization [44]. The exact function of each *Tep* in host defense remains poorly understood despite several studies and their potential role as opsonins has not been definitely determined [33,45–54]. Although an initial study failed to reveal a role of *Tep1*, *Tep2*, *Tep3*, or *Tep4* in several infection models, even when *Tep2-Tep3-Tep4* triple mutants were assayed [44], a quadruple *Tep1-Tep2-Tep3-Tep4* mutant was reported to be sensitive to some fungal and Gram-positive bacterial infections, likely because Toll pathway activation is impaired in this quadruple mutant [55]. In addition to this apparent redundancy in function, different *Tep* genes may also have pathogen-specific functions: Whereas *Tep2* mutant or *Tep4* mutant was reported to be more sensitive to infections with *Porphyromonas gingivalis* [56], they unexpectedly were more resistant to challenge with *Photorhabdus* species [49,57].

Besides the systemic and local immune responses that lead to the expression of AMPs, another humoral immune response in flies depends on the phenoloxidase activation cascade that leads to melanization at the wound site as well as to the production of reactive oxygen species [58,59]. This phenoloxidase activation cascade plays an important role in combating some fungal and Gram-positive bacterial pathogens [60,61]. The proteolytic activation cascade that ultimately leads to the cleavage of the prophenoloxidase (PPO) into active PO is initiated by the binding of pattern recognition receptors to microbes [62].

Here, we report the unexpected observation that the *P. aeruginosa rhlI* and *rhlR* null mutant phenotypes are distinct in the *Drosophila* intestinal infection model and also upon ingestion of *P. aeruginosa* by *C. elegans*. We document the function of RhlR in escaping Tep4-mediated opsonization and propose a model that gives an integrated view of Tep4 function in host defense against bacterial infections.

Results

ΔrhlI is more virulent than *ΔrhlR* in the *Drosophila* intestinal infection model

Ingested *rhlI* was less virulent than wild-type *P. aeruginosa* PA14 but more virulent than *rhlR* (Fig 1A and B). Importantly, *rhlI* proliferated in the hemolymph, although it grew less rapidly than wild-type PA14, whereas *rhlR* was cleared from the hemolymph (Fig 1C and D). Consistent with this latter result, *rhlI* but not *rhlR* triggered a systemic immune response, as monitored by measuring the expression of the *Diptericin* gene (Appendix Fig S1E and F). Similar results were obtained with independent *rhlR* and *rhlI* in-frame deletion mutants constructed by another laboratory (Appendix Fig S1) [63]. We conclude that RhlR and RhlI have differing functions in the *Drosophila* intestinal infection model.

rhlR and *rhlI* killed flies in which phagocytosis had been impaired by the prior injection of latex beads (LXB flies) much faster than PBS-injected control flies, at approximately the same rate, but more slowly than wild-type PA14 (Fig 1E and F). Does this reflect a regained virulence of the mutants in this context [25] or simply that these immunodeficient flies are more sensitive to *P. aeruginosa* intestinal infections? To answer this question, it is informative to measure the difference in LT_{50} (the time for 50% lethality) values of control versus latex bead-injected flies ($\Delta = LT_{50}[\text{wt-wt}^{LXB}]$) for each mutant and to compare it to the Δ measured for wild-type PA14: If it is equal in magnitude, then the effect is likely non-specific; if it is higher, it would suggest that the cellular immune response is targeted by the wild-type version of the mutated bacterial gene. *rhlR* did recover virulence with a Δ of 4.7 days, compared to 2.4 days for wild-type PA14 (Fig 1G), which corresponds to the level of recovered virulence reported earlier [25]. With a value of 3.5 days, *rhlI* displayed an intermediate Δ, which was significantly distinct from that measured for *ΔrhlR* but not that of wild-type PA14 (Fig 1G). Thus, we conclude that PA14 RhlR plays a predominant role in counteracting phagocytosis.

rhlI and wild-type *PA14*, but not *rhlR*, strains colonize the *C. elegans* digestive tract

We tested whether the differences that we observed in the virulence of *rhlR* and *rhlI* mutants in the *Drosophila* intestinal infection assay were reflected in the *C. elegans-P. aeruginosa* nematode "slow-killing" survival assay [21,64]. Indeed, two independently constructed in-frame *rhlR* deletion mutants in the PA14 background

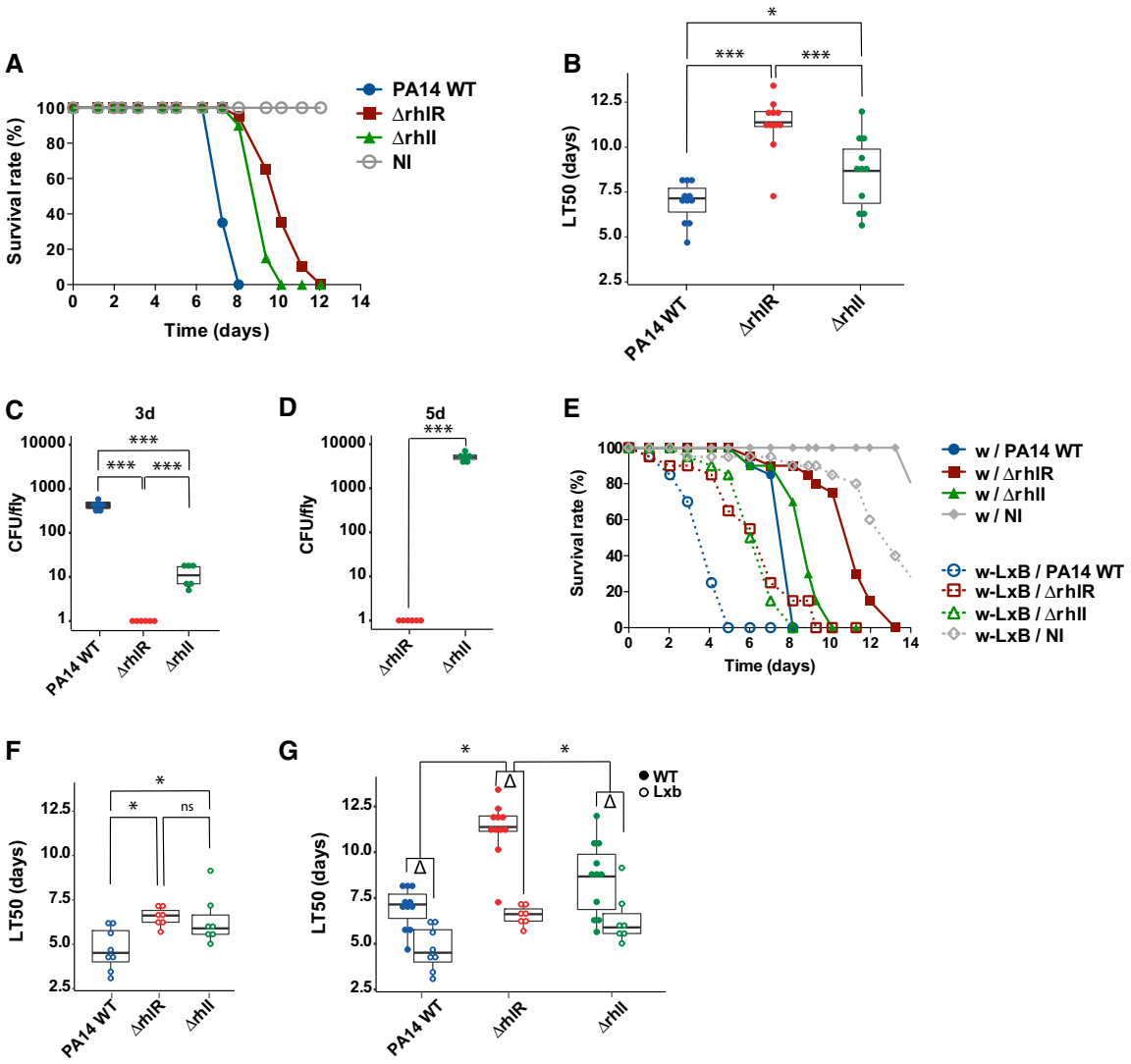

Figure 1. *RhlI* displays a distinct phenotype from that of *rhlR* in the *Drosophila* intestinal infection model.

Wild-type flies were fed on wild-type (WT) PA14 bacteria, *rhlR* (Δ*rhlR*), or *rhlI* (Δ*rhlI*).

A Representative survival curves of infected and non-infected (NI) flies. Flies died faster from the infection with PA14 WT than with *rhlR*. Flies infected with *rhlI* exhibited an intermediate survival phenotype. One representative experiment is shown. Statistical analysis of the data is shown in (B).

B Pooled LT_{50} data of wild-type flies (w^{A5001}) following intestinal infections with PA14 WT, *rhlR*, or *rhlI*. LT_{50} of flies after infection with PA14 WT was significantly lower than with *rhlR* or *rhlI* (***$P < 0.0001$ and *$P = 0.0009$, respectively). Flies were significantly more susceptible to infection with *rhlI* than with *rhlR*. The LT_{50} data from seven survival experiments are displayed (biological duplicates are also shown as there was as much variability between experiments as within experiments). ***$P < 0.001$.

C, D Bacterial titer of the hemolymph collected from flies that had ingested wild-type or mutant PA14 as indicated, three (C) or five (D) days after the ingestion of PA14 WT or ingestion of mutants as indicated. In this series of experiments, flies infected with PA14 WT had started to succumb by day 5 and were therefore not analyzed. ***$P < 0.001$.

E Survival curves of wild-type and latex bead-injected flies after intestinal infection with PA14 bacteria. In latex bead-injected flies, *rhlI* regained virulence. Note, however, that the shift in virulence was of the same amplitude as that observed for PA14 WT and contrasts with the large shift observed with *rhlR*.

F Pooled LT_{50} data of latex bead-injected flies (*w*-LXB) survival experiments. *w*-LxB flies died significantly slower after *rhlR* infection than with PA14 WT (*$P = 0.015$). A slight decrease in virulence was observed between PA14 WT and *rhlI* (*$P = 0.02$). No difference in virulence was detected between *rhlR* and *rhlI*. Data represent the LT_{50}s from five experiments (biological duplicates are also shown as there was as much variability between experiments as within experiments).

G Differences of LT_{50}s between WT flies pre-injected with PBS (WT, filled circles; these data are also shown in B) and flies pre-injected with latex beads (LXB, open circles; these data are also shown in F) after intestinal infection with PA14 WT, *rhlR*, or *rhlI*, bars indicate medians. Δ represents the difference between PBS and LXB-injected flies ($LT_{50}[wt\text{-}wt^{LXB}]$), and this difference was highly significant for all bacterial genotypes ($P = 0.0032$ for WT PA14 and *rhlI*, $P < 0.0001$ for *rhlR*; not shown on the graph for simplicity). Data represent the LT_{50}s from five experiments (biological duplicates are also shown as there was as much variability between experiments as within experiments). *$P < 0.05$.

Data information: (B–D, F, and G) Bars represent medians, the upper and lower limits of boxes indicate, respectively, the first and third quartiles and whiskers encompass data points within 1.5 times the interquartile range. Data were analyzed using linear models and reported *P*-values are relative to the post-hoc pairwise comparisons between the groups of interest.
Source data are available online for this figure.

were markedly less virulent than two independently constructed *rhlI* deletion mutants in their ability to kill *C. elegans* (Fig EV1A). We next used a quantitative assay (Fig EV1B–E, see Materials and Methods) that monitors the accumulation of live bacterial cells in the intestine of the nematodes using PA14, *rhlR,* and *rhlI* bacteria expressing GFP to monitor the level of intestinal colonization. Live wild-type *P. aeruginosa* PA14 cells start accumulating in the intestine 24–48 h post-infection. Two independent *rhlR* mutants in the PA14 background colonized the *C. elegans* intestine at significantly lower levels than two independent *rhlI* mutants. Indeed, in this colonization assay, *rhlI* was indistinguishable from wild-type PA14. An alternative explanation for these results is that *C. elegans* preferentially feeds on the *rhlI* compared to *rhlR* and simply overwhelms the immune response with a larger number of ingested cells. However, this possibility was ruled out by monitoring the pumping (feeding) rate of *C. elegans* feeding on wild-type, *rhlR,* and *rhlI*. *C. elegans* pumped at the same rate on all three strains (Appendix Fig S2). These data suggest that RhlR but not RhlI is a predominant determinant for PA14 virulence in both fly and nematode intestinal infections and that the primary effect in *C. elegans* appears to be on colonization.

Phagocytosis protects *Drosophila* against invasion of its hemocoel by wild-type PA14 during the early phase of the infection

In our *Drosophila* intestinal infection models, flies constantly feed on the pathogen present on a filter. Using bacteria expressing different colored fluorescent proteins, we previously showed that *S. marcescens* continuously crosses the intestinal barrier during the infection [26]. Figure 2A and B shows that when flies that had been feeding on dsRed-labeled PA14 bacteria for 4 days were switched to a filter laced with GFP-labeled PA14, the green bacteria progressively replaced the red bacteria both in the gut and in hemocoel compartments. We conclude that *P. aeruginosa*, like *S. marcescens*, continuously crosses the intestinal barrier during the infection.

Next, we asked at what time periods during an infection is phagocytosis important in preventing PA14 growth in the hemolymph. To this end, we saturated the phagocytic apparatus of hemocytes by injecting latex beads into flies at different time points during infection. As expected, blocking phagocytosis 1 day prior to the infection led to an earlier demise of the PA14-infected flies compared to PBS-injected control flies. Similar results were found when latex beads were injected 4 h or 1 day after infection, although in the latter case, the difference was not significant (its value was nevertheless similar to that obtained by injection 1 day prior to infection; Fig 2C). The injection of latex beads 4 or 6 days after the beginning of the ingestion of wild-type PA14 did not affect the survival rate of flies. That is, the LT_{50} values were similar to those of control (PBS-injected) flies, consistent with the conclusion that starting about 4 days after infection the cellular immune response no longer plays a major role in limiting a wild-type PA14 infection.

In contrast to wild-type PA14, *rhlR* was kept in check by phagocytosis at least up to day four and to some extent up to 6 days after infection (Fig 2D and F). Phagocytosis was efficient against *rhlI* for approximately 4 days, which displayed again an intermediate phenotype (Fig 2E and F). These data suggest that hemocytes constantly keep in check bacteria that have crossed the intestinal barrier, but that as a consequence of RhlR function, wild-type PA14 ultimately escapes this immune surveillance.

A recent study has reported that hemocytes are recruited to the gut after the ingestion of bacteria [65]. We confirmed this finding in the case of *P. aeruginosa* oral infection, with a significant recruitment observed at 4 h after the beginning of the infection with either wild-type PA14, *rhlR,* or *rhlI* mutants (Fig 2G and H, Appendix Fig S3). While hemocytes remained associated with the midgut for at least 3 days after the beginning of the ingestion of wild-type PA14 or *rhlR* bacteria, they were not in the case of *rhlI* bacteria (Fig 2H). While some ingested bacteria could be detected in the hemocytes recruited to the gut, this phenomenon was not reproducible enough

Figure 2. Phagocytosis is required during the early stage of the infection in *Drosophila*.

A, B Wild-type *Drosophila* were orally infected with wild-type PA14 expressing dsRed (PA14-dsRed). After 4 days, infected flies were transferred to tubes containing wild-type PA14 expressing GFP (PA14-GFP). At day 5 of the infection (1 day after transferring flies to PA14-GFP), most PA14 bacteria in the *Drosophila* gut expressed dsRed and only 10% expressed GFP (A). However, at day 6 (2 days after the transfer of flies to PA14-GFP), only GFP-positive bacteria were detected in the gut. Results were similar for bacteria retrieved from the hemolymph compartment (B). Each dot corresponds to a sample of 10 flies.

C–E LT_{50}s from survival experiments of WT flies (ω) after intestinal infection with PA14 WT (C), *rhlR* (D), or *rhlI* (E) mutants and injection of either latex beads (LXB, open circles) or PBS (filled circles) at different time points of the infection. Latex beads or PBS was injected either 1 day before the infection started (−1 day) or 4 h (+4 h), 1 day (+1 day), 4 days (+4 days), or 6 days (+6 days) after the infection started. Black filled circles correspond to the survival of infected, uninjected flies. (C) LT_{50}s of w^{A5001}-LxB were significantly lower than w^{A5001} only at −1 day (**P = 0.003) and +4 h (*P = 0.013). (D) LT_{50}s of ω-LXB flies were significantly lower than ω at most times during the infection (−1 day: ***$P = 5 \times 10^{-5}$, +4 h: ***$P = 3 \times 10^{-6}$, and +4 day: **P = 0.01). (E) A similar phenotype was observed with flies infected with *rhlI* (−1 day: **P = 0.002, +4 h: **P = 0.006, +1 day: ***$P = 8 \times 10^{-6}$, and +4 days: *P = 0.03). Note, however, that for injections of latex beads at day 4, the difference was reduced, as compared to earlier time points of injection of latex beads. The cumulative LT_{50} data from at least three experiments (only two experiments for *rhlI*) are shown, except for day 6.

F Δ: Differences of LT_{50}s between WT flies injected with PBS (WT) and flies injected with latex beads (WT^{LXB}) after intestinal infection with PA14 WT, *rhlR*, or *rhlI* in at least two experiments. *$P < 0.05$; ***$P < 0.001$.

G Guts of transgenic flies with GFP-labeled hemocytes were dissected in a manner that preserves the association of hemocytes with the digestive tract and were examined by fluorescence confocal microscopy. Green: GFP; blue: DAPI staining of nuclei. Scale bars: 100 μm.

H Analysis of hemocytes recruited to the fly intestine upon infection with either wild-type PA14, *rhlR*, or *rhlI*. All three strains elicited recruitment of hemocytes to the gut (4 h after the beginning of the infection, for each bacteria ***$P < 0.0001$); at 3 days after the beginning of the infection, there were fewer hemocytes recruited after infection with *rhlI* (**P = 0.0025 between *rhlR* and *rhlI*). Data represent three pooled experiments. *$P < 0.05$.

Data information: (C–F and H) Bars represent medians, the upper and lower limits of boxes indicate, respectively, the first and third quartiles and whiskers encompass data points within 1.5 times the interquartile range. Data were analyzed using linear models and reported P-values are relative to the post-hoc pairwise comparisons between the groups of interest.
Source data are available online for this figure.

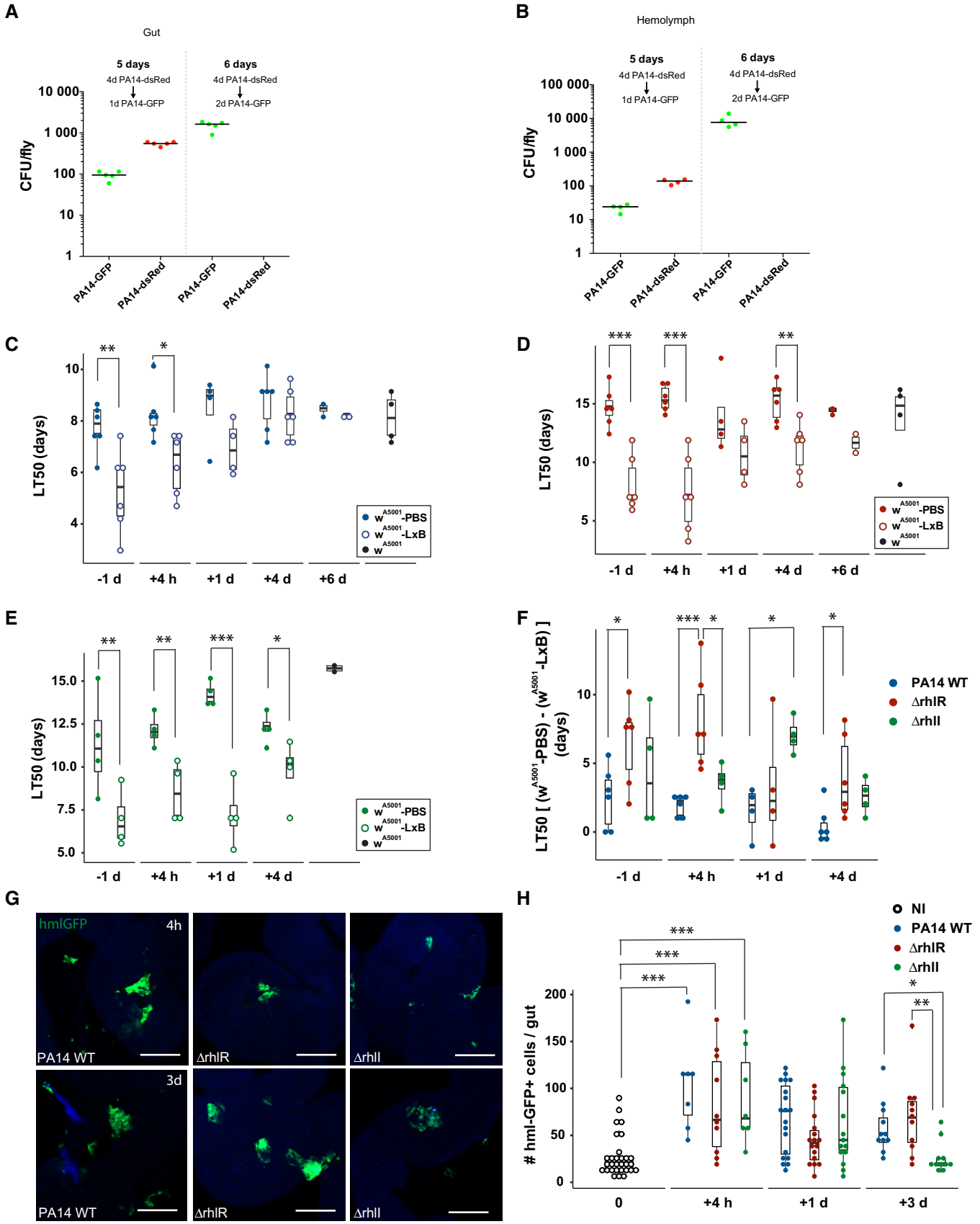
A
Gut
5 days
4d PA14-dsRed
1d PA14-GFP
6 days
4d PA14-dsRed
2d PA14-GFP
CFU/fly
PA14-GFP
PA14-dsRed
PA14-GFP
PA14-dsRed
B
Hemolymph
C
LT50 (days)
-1 d
+4 h
+1 d
+4 d
+6 d
D
E
F
LT50 [(w-PBS) - (w-LxB)] (days)
PA14 WT
ΔrhlR
ΔrhlI
G
hmlGFP
4h
3d
PA14 WT
ΔrhlR
ΔrhlI
H
NI
hml-GFP+ cells / gut
0
+4 h
+1 d
+3 d

Figure 2.

to allow reliable quantification of their uptake and thus to assess whether this recruitment of hemocytes to the gut may be involved in controlling bacteria that have crossed the intestinal barrier.

Drosophila Tep4 is required for host defense against ingested PA14

Given the importance of phagocytosis in controlling bacteria that have escaped from the digestive tract, we asked whether Teps might not be involved in this process as opsonins. We tested mutations affecting the *Tep2*, *Tep3*, and/or *Tep4* genes [44] in the intestinal infection assay. Only *Tep4* and a triple *Tep2-Tep3-Tep4* mutant displayed an increased susceptibility to PA14 ingestion (Fig 3A and D). Of note, uninfected *Tep3* mutants fed on a sucrose solution displayed an enhanced fitness when compared to wild-type or other *Tep* mutant lines, which may contribute to its apparent decreased susceptibility to ingested PA14 (Appendix Fig S4C, Fig 3A and B). In the case of *Tep1*, since no mutants were available, we tested an

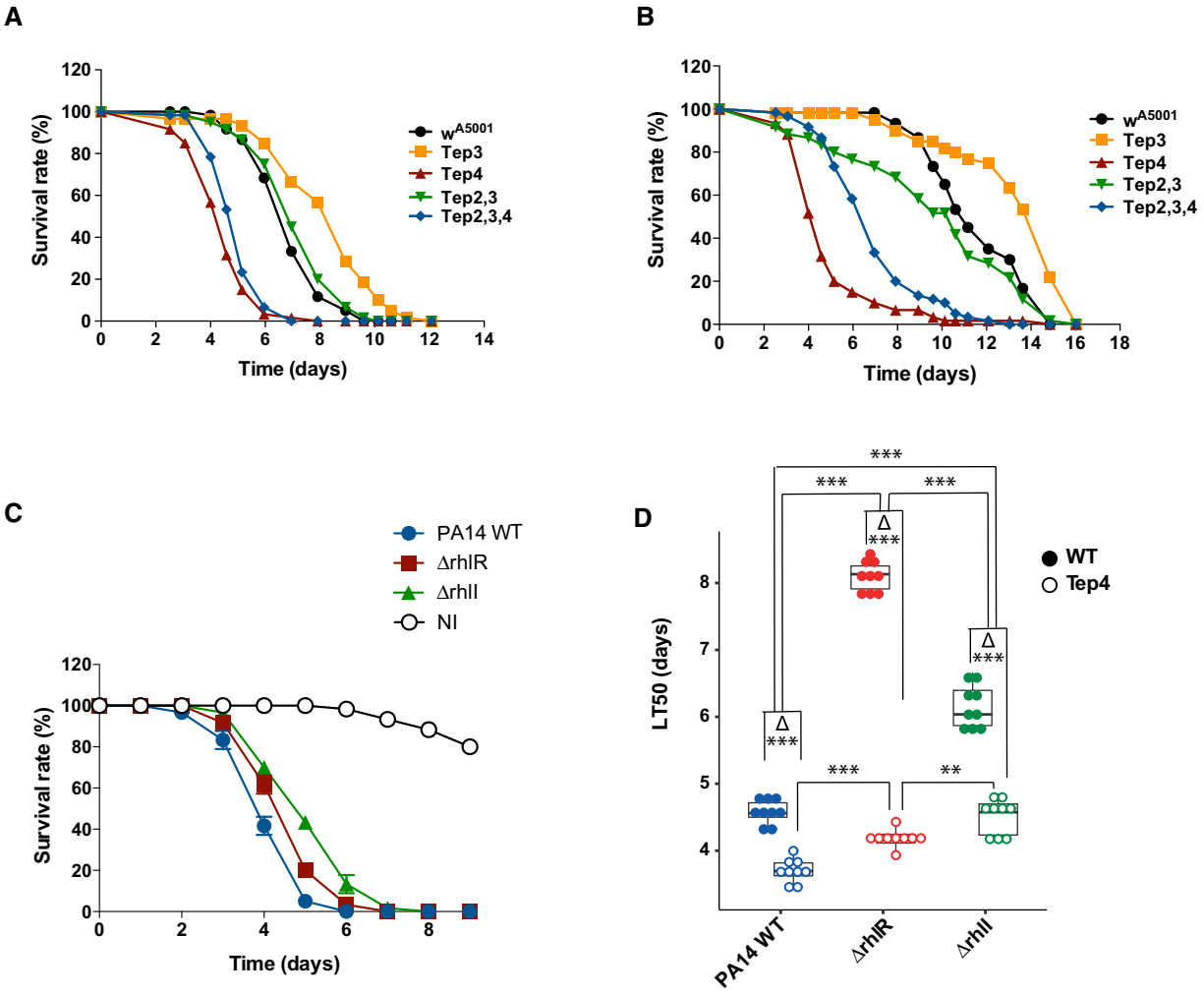

Figure 3. *RhlR* circumvents *Tep4*-mediated host defense.

A, B *Drosophila* wild-type flies (w^{A5001}), single *Tep3* or *Tep4* mutants, the double *Tep2,3* mutant, and the triple *Tep2,3,4* mutant were orally infected with PA14 WT (A) or *rhlR* (B) in parallel survival experiments. (A) *Tep4* and *Tep2,3,4* mutant flies were significantly more susceptible to PA14 infection compared to w^{A5001}. No difference in survival was detected between the *Tep2,3* mutant and w^{A5001}. Surprisingly, *Tep3* mutants seemed to be more resistant to infection. (B) A strong enhancement of *rhlR* virulence was observed with the *Tep4* and *Tep2,3,4* mutants compared to w^{A5001} flies. *Tep2,3* and w^{A5001} exhibited nearly the same rate of death when challenged with *rhlR*. The *Tep3* mutant seemed again to be more resistant to the infection. One representative experiment out of three (each with biological triplicates, except for uninfected flies) is shown.

C The survival of *Tep4* flies infected with PA14 WT, *rhlR*, or *rhlI* was examined. One representative experiment out of three (each with biological triplicates) is shown.

D Quantification of the experiments shown in (C), which had been performed in parallel with w^{A5001} flies; filled circles: wild-type flies, open circles: *Tep4*. The triplicates were analyzed as independent experiments as there was as much variability between experiments as within experiments. All differences between wild-type flies and the *Tep4* mutant (Δ) were highly significant (***$P < 0.0001$). Bars represent medians, the upper and lower limits of boxes indicate, respectively, the first and third quartiles and whiskers encompass data points within 1.5 times the interquartile range. Data were analyzed using linear models and reported *P*-values are relative to the post-hoc pairwise comparisons between the groups of interest. **$P < 0.01$; ***$P < 0.001$.

Source data are available online for this figure.

RNAi transgene expressed either in hemocytes or in the fat body; however, we did not observe any change in the virulence of ingested PA14 in the *Tep1* knockdown (Appendix Fig S4A and B). We conclude from these data that Tep4, but not other thioester-containing proteins, is required for host defense against ingested PA14.

Next, we found that *rhlR* became almost as virulent as wild-type PA14 only when ingested by *Tep4* or *Tep2-Tep3-Tep4* mutants (Fig 3A, B and D). *rhlR* recovered virulence to a much larger extent than *rhlI* when ingested by *Tep4* flies (Fig 3C; compare the Δ for *rhlR* and *rhlI*, Fig 3D). Interestingly, the injection of latex beads in *Tep4* flies only modestly increased the virulence of *ΔrhlR* bacteria when compared to PBS-injected *Tep4* flies (Appendix Fig S4D and E), suggesting that phagocytosis of *rhlR* is already severely affected in the *Tep4* mutant. Hence, the behavior of the *rhlR* mutant is similar in *eater*, which encodes a putative phagocytic receptor [27], and *Tep4* mutant flies [25], thereby raising the possibility that both fly genes are involved in the same process. These data suggest that a RhlR-dependent process bypasses the Tep4-mediated control of *P. aeruginosa* present in the hemocoel.

Phagocytosis of PA14 bacteria is impaired in *Tep4* mutant hemocytes

To quantitatively monitor the uptake of PA14, we used assays that relied on larval hemocytes. First, we injected heat-killed, pHrodo®-labeled bacteria in wild-type or *Tep4* third-instar larvae. After 45 min, the larvae were bled and a phagocytic index was established. When analyzed globally by ANOVA, the data showed that wild-type hemocytes ingested significantly more bacteria than *Tep4* hemocytes ($P = 0.004$), but when comparisons were made according to the genotype of the phagocytosed bacteria, a significant difference was detected for wild-type PA14 but not for *rhlR* (Fig 4A). Similarly, a global analysis revealed significant differences between heat-killed wild-type PA14 and *rhlR* uptake by hemocytes ($P = 0.01$), yet a difference between these two bacterial genotypes was significant only for *Tep4* hemocytes (Fig 4A). This was not necessarily unexpected as heat-killing likely inactivates *rhlR*-dependent virulence factors and might also alter the surface of bacteria.

We therefore modified the assay by using live bacteria [66] and an antibody we had raised against PA14 to differentially immunostain the bacteria, prior to and after permeabilization of the fixed cells. As before, *Tep4* hemocytes exhibited a decreased uptake of bacteria compared to wild-type hemocytes when analyzed globally ($P = 0.0002$); however, in the case of the comparisons by bacterial genotypes, only the difference for wild-type PA14 was significant (Fig 4B). When analyzing the data globally according to bacterial genotypes, a significant difference was found between wild-type PA14 and *rhlR* ($P = 0.0005$) but not between wild-type PA14 and *rhlI* nor between *rhlR* and *ΔrhlI*. When comparing wild-type PA14 and *rhlR* specifically per fly genotype, the difference between these two strains was significant only in the *Tep4* mutants. Upon repeating this experiment in wild-type flies using bacterial strains obtained from

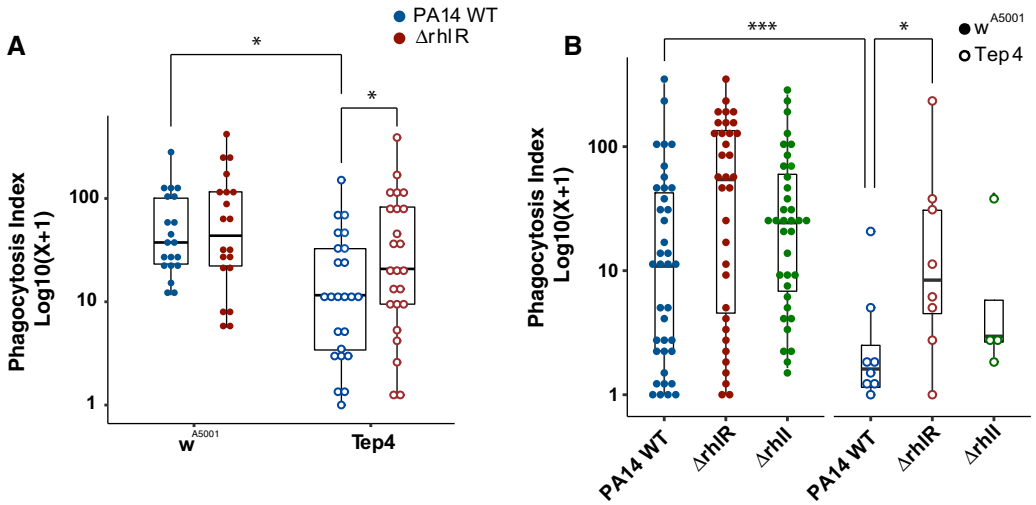

Figure 4. Tep4 is required for phagocytosis of *rhlR* mutant bacteria.

A Heat-killed pHrodo®-labeled bacteria of the indicated genotype were injected into either wild-type or *Tep4* third-instar larvae and incubated for 45 min. The hemocytes were then retrieved. Bacteria present in phagosomes were fluorescent and used to measure the phagocytic index. *rhlR* bacteria injected into *Tep4* (open circles) were significantly more phagocytosed than wild-type bacteria (filled circles) ($P = 0.02$); PA14 WT were more readily phagocytosed by wild type than by *Tep4* hemocytes (*$P = 0.01$).

B The experiment is similar to that shown in (A), except that live bacteria were used and a differential antibody staining procedure was performed to reveal phagocytosed bacteria: Phagocytosed bacteria were stained only after permeabilization and fluoresced green whereas non-ingested bacteria were stained by both secondary antibodies, red, and green, which were, respectively, used prior and after the permeabilization step (see Materials and Methods section). PA14 WT, *rhlR*, or *rhlI* were injected in wild-type or *Tep4* larvae. Again, PA14 WT was more readily phagocytosed by wild-type than by *Tep4* larval hemocytes (***$P = 0.0006$). *rhlR* were engulfed more efficiently than PA14 WT bacteria by *Tep4* hemocytes (*$P = 0.01$).

Data information: Bars represent medians, the upper and lower limits of boxes indicate, respectively, the first and third quartiles and whiskers encompass data points within 1.5 times the interquartile range. Data were analyzed using generalized linear models assuming a negative binomial distribution and reported *P*-values are relative to the post-hoc pairwise comparisons between the groups of interest.
Source data are available online for this figure.

another laboratory, we observed that *rhlR* bacteria were significantly better phagocytosed than either wild-type PA14 or *rhlI* (Appendix Fig S1G). In summary, both assays revealed that wild-type flies ingest wild-type PA14 better than *Tep4* mutant flies and that *rhlR* appears to be better phagocytosed than wild-type PA14 in *Tep4* mutant flies. Both phagocytosis assays, however, failed to discriminate between *rhlR* and *rhlI*, possibly because they are not sensitive enough.

Tep4 opsonizes *rhlR* better than *rhlI* or wild-type bacteria

We next designed an experiment to assess whether Tep4 functions as an opsonin, that is, that it is deposited on the surface of bacteria to facilitate its detection and subsequent ingestion by hemocytes (Fig 5A). Wild-type PA14 was poorly phagocytosed in this assay (medians of phagocytic index lower than 10), whether the bacteria

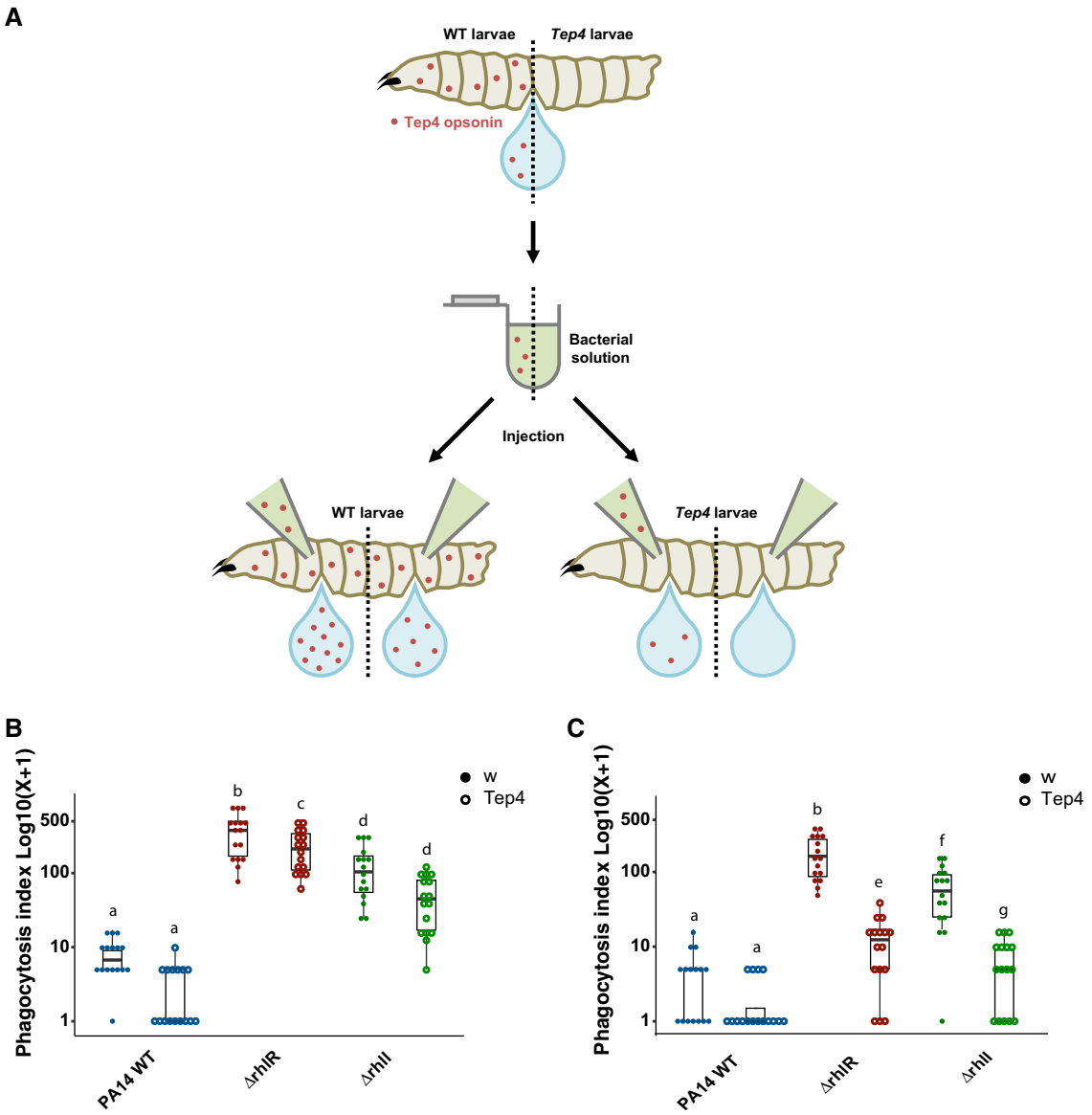

Figure 5. Tep4 is an opsonin that preferentially detects *rhlR* over *rhlI* mutant bacteria.

A Scheme of the experimental procedure. Live bacteria were incubated with either wild-type or *Tep4* hemolymph and were thereafter injected into naive larvae, which were either wild-type or *Tep4*. The phagocytosis index was then measured as in Fig 4B.

B, C Bacteria pre-incubated with wild-type or *Tep4* hemolymph are represented in pairs, respectively, with filled (left) and open (right) circles. PA14 WT, *rhlR*, or *rhlI* bacteria pre-incubated with either wild-type (wt) or *Tep4* hemolymph were injected into wild-type larvae (B) or into *Tep4* larvae (C). Data were analyzed using generalized linear models assuming a negative binomial distribution and statistically homogeneous groups (indicated by letters) were defined based on the post-hoc pairwise comparisons between the different groups. The difference between each group is highly significant ($P < 0.001$). Bars represent medians, the upper and lower limits of boxes indicate, respectively, the first and third quartiles and whiskers encompass data points within 1.5 times the interquartile range.

Source data are available online for this figure.

were initially incubated in the hemolymph of Tep4-containing wild-type larvae or *Tep4* larvae and then secondarily injected into wild-type (Fig 5B) or *Tep4* (Fig 5C) larvae. When these experiments were performed using *rhlR* bacteria that were injected into *Tep4* recipient larvae (Fig 5C), it made a major difference whether these mutant bacteria had initially been pre-exposed to wild-type or *Tep4* hemolymph. Tep4-dependent opsonization led to a massive uptake of bacteria (median phagocytic value of 160), whereas non-opsonized bacteria (pre-incubated with *Tep4* mutant hemolymph) were hardly ingested (median phagocytic value of 10) when the treated bacteria were injected into larvae. As expected, initially non-opsonized bacteria that were then injected in wild-type recipients (Fig 5B) were much better phagocytosed (median phagocytic value of 210), presumably because Tep4 was circulating in the hemolymph of wild-type recipient larvae. They were nevertheless ingested less efficiently than opsonized bacteria (median phagocytic value of 380; Fig 5B). Finally, *rhlI* bacteria were also opsonized in a Tep4-dependent manner, but significantly less than *rhlR* bacteria (Fig 5B: $P = 0.0005$ for opsonized bacteria, $P = 0.0008$ for non-opsonized bacteria; Fig 5C: $P < 0.0001$ for opsonized bacteria). Again, they displayed a phenotype that was intermediate to that of wild-type PA14 on the one hand, and *rhlR* on the other, although it was quantitatively closer to that of *rhlR* than that of wild-type PA14. Of note, *rhlR* and *rhlI* bacteria behaved similarly in this assay when Tep4 was entirely absent (non-opsonized bacteria in *Tep4* recipient larvae (Fig 5C)). We conclude that RhlR plays a major role in eluding Tep4-mediated opsonization of wild-type PA14. Unexpectedly, RhlI also significantly contributes to this process, even though it does not play a predominant role in virulence.

RhlR may function cell autonomously

RhlR either functions at the level of the bacterial cell in which it is expressed, for example, by altering the structure of the cell wall to prevent detection by Tep4 or, alternatively, it might control the expression of secreted factors such as proteases that would inactivate Tep4 in a non-cell-autonomous process. To discriminate between these two possibilities, we performed the opsonization assay described above on a 1:1 mix of wild-type PA14 and *rhlR* and compared their ingestion profiles to that of either strain alone subjected to the assay. Figure 6A shows that when mixed, wild-type PA14 was unable to rescue the Tep4-sensitivity phenotype of *rhlR* bacteria. A comparison of the profiles of phagocytosed bacteria (Fig 6B) supports the conclusion that the ingested bacteria in the mix are predominantly *rhlR* and not wild-type PA14. The cell-autonomous function of RhlR is in keeping with the observation that hemocytes remain able to phagocytose *Escherichia coli* even late during the infection [25].

Tep4 plays an adverse role in a PA14 systemic infection model in *Drosophila*

A recent study has reported that *Tep4* mutants are more resistant than wild-type flies to a systemic infection with the entomopathogenic bacterium *Photorhabdus luminescens* in a septic

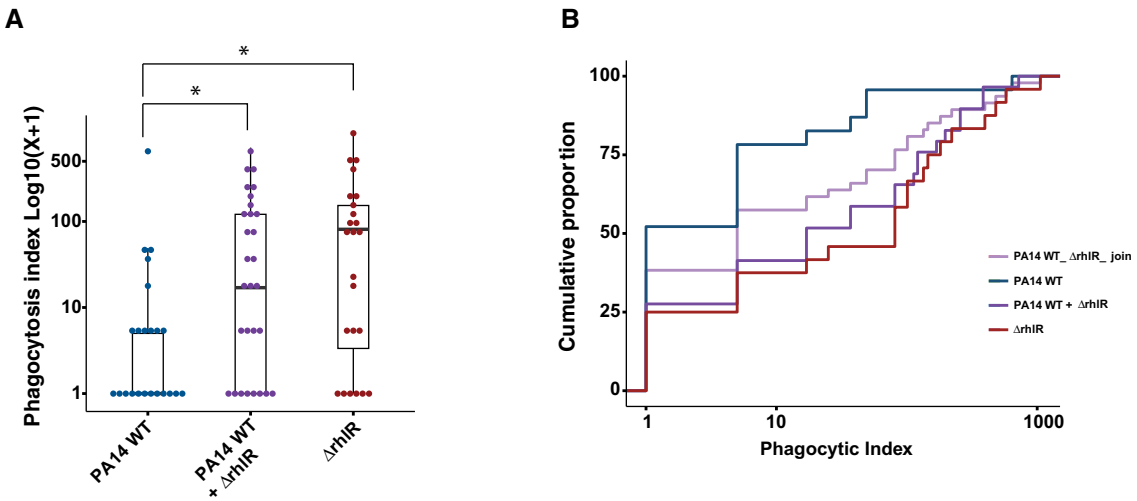

Figure 6. RhlR appears to function cell autonomously in eluding opsonization.

A, B PA14 WT, *rhlR*, or a 1:1 mix thereof was submitted to the opsonization assay using wild-type larvae as donors and recipients. Panel (A) displays the measured phagocytic indexes: PA14 WT is hardly ingested by larval hemocytes, whereas *rhlR* and the mixture of wild-type and *rhlR* bacteria display a similar distribution, as shown in (B), where the cumulative distribution of phagocytic indices is shown. The purple curve (PA14_WT_ΔrhlR_join) shows the distribution that would be obtained by pooling the data from *rhlR* and PA14 WT alone in this opsonization assay. The observed distribution of the phagocytic indices in the mix is clearly closer to that of *rhlR* (two-sample Kolmogorov-Smirnov test, $P = 0.98$), suggesting that the ingested bacteria are mostly of the *rhlR* genotype. Bars represent medians, the upper and lower limits of boxes indicate, respectively, the first and third quartiles and whiskers encompass data points within 1.5 times the interquartile range.

Data information: (A) Data were analyzed using generalized linear models assuming a negative binomial distribution and reported *P*-values are relative to the post-hoc pairwise comparisons between the different groups. *$P < 0.05$.
Source data are available online for this figure.

injury model [57]. By injecting several doses of PA14, from 10 to 1,000 CFUs, directly into the thorax of flies, we also found that *Tep4* mutants survived better than wild-type flies in this systemic infection model (Fig 7A). This difference in survival between *Tep4* and wild-type flies was no longer observed when *rhlR* was injected, thereby establishing again a relationship of altered virulence of these bacteria in a *Drosophila Tep4* mutant background (Fig 7B). Using the steady-state expression of the antibacterial peptide gene *Diptericin* as a read-out of the activation of the IMD pathway that regulates the systemic immune response against Gram-negative bacteria, we found no consistent difference of expression between wild-type and *Tep4* (Fig 7C). As a higher level of induction of the IMD pathway is unlikely to account for the increased resistance of *Tep4* mutants against PA14 infection, we tested whether the phenoloxidase cascade was more efficiently activated in this mutant background, as previously reported [57]. Indeed, we found that prophenoloxidase was more extensively cleaved in *Tep4* than in wild-type flies (Fig 7D). These data suggest that injected wild-type PA14 bacteria elude detection by the factors that trigger the phenoloxidase cascade and that Tep4 plays a role in this process of evasion from the melanization response.

Discussion

In this study, we analyzed the interactions of *P. aeruginosa* with *Drosophila* from the dual perspective of both pathogen and host. Our data lead us to propose a model in which the *P. aeruginosa* quorum-sensing regulator RhlR plays a pivotal role in virulence by diminishing the ability of the cellular immune arm of the host defense response to detect *P. aeruginosa* once the bacteria have reached the internal body cavity of the insect in an intestinal infection model. Surprisingly, the function of RhlR in eluding opsonization by Tep4 is at least partially independent of RhlI, the enzyme that synthesizes C4-HSL, the quorum-sensing signaling molecule that corresponds to RhlR. We propose that this novel C4-HSL-independent function of RhlR is to modify the bacterial surface, thereby altering the detection of *P. aeruginosa* by the immune system through Tep4. Whereas Tep4 has a protective function in this intestinal infection model, it plays a detrimental role in *Drosophila* host defense in a septic injury model, thereby underscoring that the efficiency of specific host defenses depends on the infection route and virulence strategy of the pathogen.

A rhlI-independent function of *rhlR*

Null *rhlI* and *rhlR* mutants display distinct virulence phenotypes as monitored in survival experiments in both the *Drosophila* and the *Caenorhabditis* intestinal infection models. The virulence of *rhlI* bacteria is moderately impaired whereas that of *rhlR* is severely affected. In the nematode worm, the strongly attenuated virulence of *rhlR* likely reflects its decreased ability to colonize the intestinal tract. In the fly, systemic bacteremia is thought to cause the demise of the host: Bacteremia in the hemolymph is delayed in the case of *rhlI* infection but does not occur for *rhlR*, thereby highlighting a major phenotypic difference between the two mutants. Thus, *rhlR* appears to have a *rhlI*-independent function *in vivo*. In support of this conclusion, a recent study by Mukherjee *et al* [67] showed that *rhlI* and *rhlR* mutants exhibit different colony biofilm morphologies, that *rhlI* mutants are more virulent than *rhlR* mutants in a mouse infection model, and that there are three categories of *rhlR*-dependent regulons that require *rhlI* function to varying degrees. The Mukherjee *et al* [67] study also established that RhlR activity *in vitro* depends on a second ligand, the nature of which remains elusive at this stage. It is unknown whether a ligand distinct from C4-HSL is also required for virulence in flies, for instance, to stabilize the folding of the hydrophobic core of the RhlR regulators, a role thought to be assumed by HSL for LuxR regulators [3]. Of note, RhlR does not appear to be activated by 3-oxo-C12-HSL, the LasR ligand [67]. Furthermore, *lasR* and *lasI* mutant bacteria display only a modestly decreased virulence phenotype in the *Drosophila* intestinal infection model (Appendix Fig S5). Because phagocytosis and *Tep4*-mediated detection represent an efficient host defense at the very beginning of the infection when only few bacteria can be retrieved from the hemolymph, it seems unlikely that an alternative RhlR ligand is expressed in a quorum-sensing-dependent manner but rather as the result of the specific conditions the bacteria encounter in the host hemolymph or when going through its digestive tract.

Because *rhlR* function is required at a stage in which only a few bacteria can be retrieved, it is technically difficult to measure gene expression in this context and specific reporter transgenes will be needed to assess whether *rhlR*-dependent-*rhlI*-independent genes are expressed. Nevertheless, because of the similarities observed between the fly intestinal infection model and the nematode slow-killing infection paradigm observed in this study, we used the *Drosophila* intestinal infection model to screen more than 300 PA14 mutant lines that had been identified as less virulent in *C. elegans* [68]. This approach, however, failed to identify mutants, or combinations thereof, with as strong a phenotype as *rhlR* (Haller *et al*, in preparation). Thus, this approach appears to have failed to identify any gene(s) encoding the enzyme synthesizing a putative second RhlR ligand.

RhlR counteracts the cellular host defense by eluding detection by Tep4

Our phagocytosis and opsonization data are consistent with a model in which RhlR controls the expression of gene products that mask the site being recognized by Tep4 or a Tep4-associated protein, presumably on the bacterial cell wall. Alternatively, RhlR may actively inhibit the uptake of opsonized bacteria, for instance by impeding the cytoskeletal rearrangements in hemocytes that are necessary for phagocytosis, but this process would not involve factors secreted in the hemolymph.

Insect thioester-containing proteins belong to the complement family and have been shown to be involved in the opsonization of bacteria in mosquitoes. The deletion of four *Drosophila* Teps did not render the flies susceptible to infection with non-pathogenic Gram-negative bacteria [55]. Tep2 has been reported to be required for the uptake of *E. coli*, a Gram-negative bacterium, by cultured S2 cells [48], a finding confirmed *in vivo* [49]. In contrast, we find no involvement of Tep2 in our *in vivo* intestinal infection model with *P. aeruginosa*, at least in terms of virulence, but do detect a requirement for Tep4 in phagocytosis and opsonization assays. Given that the structure of mosquito

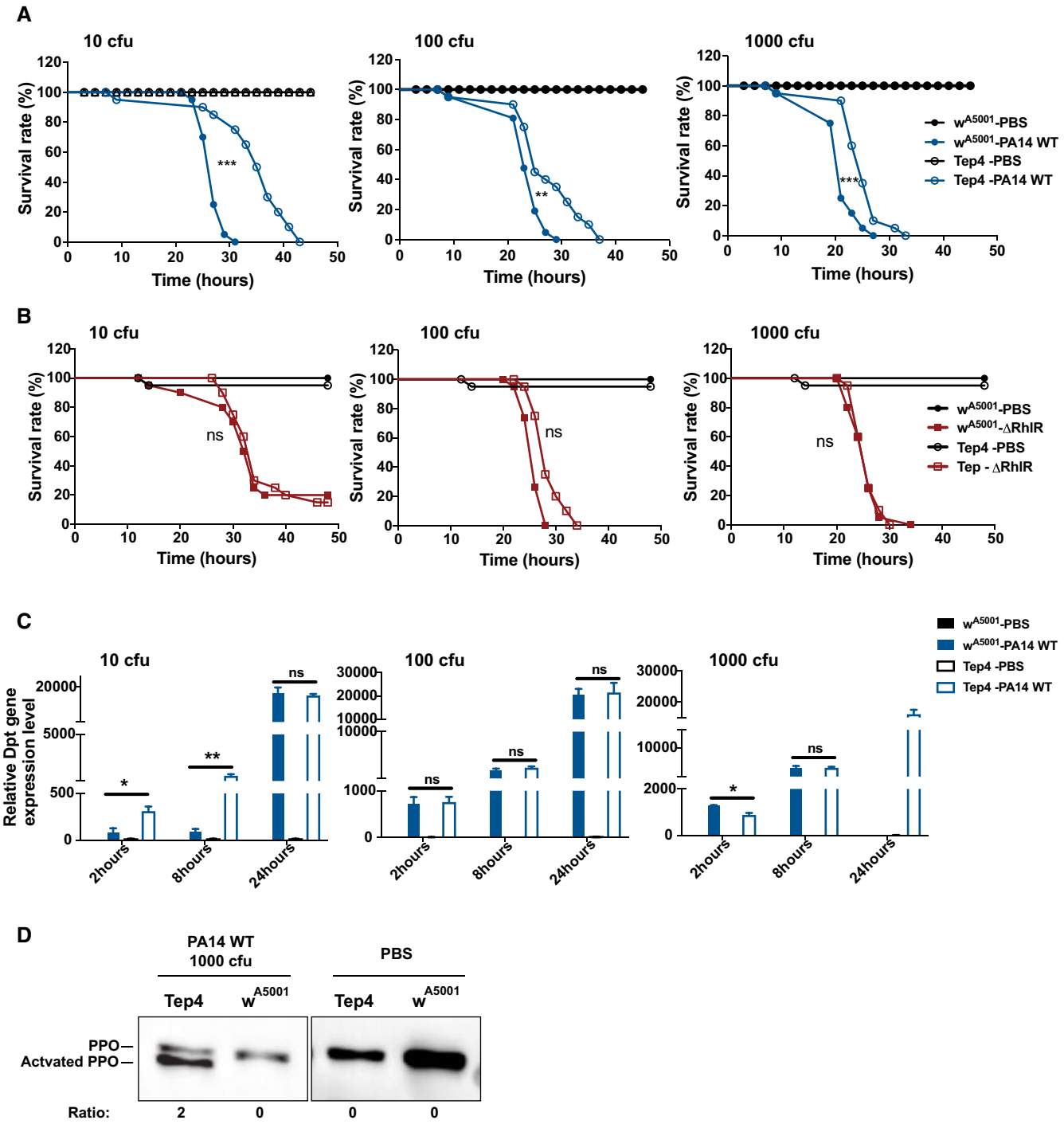

Figure 7. *Tep4* mutants are more resistant to a systemic infection with PA14.

A, B The survival of wild-type and *Tep4* flies was examined, after injection of PBS as a non-infected control (NI), or 10, 100, 1,000 CFUs of PA14 WT (A) or *rhlR* (B). One representative experiment out of 10 (A) or out of three (B) is shown. $^{**}P < 0.01$; $^{***}P < 0.001$. A log-rank test was used.

C *Diptericin* expression was measured by RT–qPCR in wild-type and *Tep4* flies at 2, 8, or 24 h after injection of PBS (NI), or of 10, 100, 1,000 CFUs of PA14 WT bacteria. Each experiment was performed independently three times, and a representative experiment is shown. The *Diptericin* expression shown is relative to *RpL32* expression and normalized to the non-infected wild-type control. Error bars represent the standard deviation. $^{*}P < 0.05$; $^{**}P < 0.01$ (unpaired *t*-test).

D The cleavage of the prophenoloxidase was analyzed 2 h after injection of 1,000 CFUs of PA14 WT bacteria or PBS, by Western blotting using a pan-phenoloxidase antibody. The intensity of the prophenoloxidase (PPO) and of the cleaved active phenoloxidase (activated PO) bands was measured, and the ratio of the measurements is shown below the blot. This experiment was performed twice with a similar result.

Source data are available online for this figure.

thioester-containing protein 1 is similar to that of complement family C3 [34], a well-described opsonin, our data are compatible with a model of direct opsonization of bacteria by Tep4. Why Tep4 and not other Teps mediate this effect will require further investigations and a better understanding of the exact mechanism of action of *Drosophila* Teps.

It is striking that the opsonization phenotype of *rhlI* is quantitatively closer to that of *rhlR* than that of wild-type PA14, which appears to be poorly opsonized. In contrast, both wild-type PA14 and *rhlI* ultimately cause a systemic bacteremia while *rhlR* is cleared from the hemolymph. Thus, a relatively minor difference in opsonization efficiency translates to a major effect with respect to the establishment of a systemic infection. This observation underscores the critical importance of opsonization levels in controlling pathogenic bacteria that escape from the digestive tract. Alternatively, the difference of virulence observed between *rhlI* and *rhlR* would not be linked to opsonization.

The opposite roles of *Tep4* in host defense against the same pathogen according to the infection route

The finding that Tep4 plays a protective function in the intestinal infection model whereas it is detrimental in the case of a direct systemic infection is paradoxical. This may actually represent two faces of the same phenomenon. *P. aeruginosa* may have developed a stealth strategy to avoid detection by the immune system and thus may actively hide any features that might reveal its presence. We propose here that RhlR plays a critical role in a program that allows *P. aeruginosa* to function in a stealth mode that does not activate an immune response. As a result of RhlR action, only a few sites would be available on the surface of the wild-type bacteria for Tep4 direct or indirect binding. There, Tep4 would mediate opsonization and then subsequent phagocytosis of the bacteria. Our data in the systemic infection model are compatible with the possibility that Tep4 competes for these sites with pattern recognition receptors (PRRs) that activate the phenoloxidase cascade since PO activation occurs efficiently only in the *Tep4* mutant background. It is likely that small peptidoglycan (PGN) fragments released by proliferating bacteria represent a major trigger of the IMD pathway in addition to large PGN fragments directly sensed by PGRP-LC, thereby accounting for the apparent normal expression of *Diptericin* when flies are challenged with injected PA14. In contrast, we have previously established that some fungi and Gram-positive bacteria trigger the phenoloxidase cascade through defined PRRs [62]. The situation is less clear as regards Gram-negative bacteria. While the original characterization of PGRP-LE suggested that it triggers the phenoloxidase activation cascade [69] and acts non-cell autonomously [70], subsequent studies documented a role for PGRP-LE as an intracellular sensor for PGN fragments [28,71,72]. Thus, further work will be required to identify whether Tep4 actually competes with PRRs in the detection of pathogens. A major challenge will be to establish how RhlR influences the surface properties of PA14 or alternatively inhibits the uptake of opsonized bacteria.

Our results with *Tep4* further suggest that the cellular immune response is a relevant defense when a few bacteria enter the hemocoel after escaping from the digestive tract in the intestinal infection model, but not in the septic injury model. Conversely, melanization mediated by activated phenoloxidase appears to be somewhat effective after injection of PA14. Thus, infection by different pathogens challenges the immune system in unique ways that depend not only on the pathogen but also on its infection route. Thus, depending on the pathogen and mode of infection, some host defenses may be important whereas others might be bypassed or neutralized or worse, become detrimental by attenuating the action of the appropriate defense responses.

Finally, *rhlR* bacteria exhibit reduced dissemination capacities in a rodent lung infection model when compared to *rhlI* or wild-type PA14 [67]. By analogy to our findings in the *Drosophila* intestinal infection model, it will be interesting to determine whether the complement system restricts the systemic escape of *rhlR* from the mouse lung into the periphery.

Materials and Methods

Many of the methods employed in this study were described in detail in Haller *et al* [73].

Drosophila stocks and culture

The following strains were used: w^{A5001} as wild type [74], and the mutants *Tep3* (d03976) [74], *Tep4* (EY04656) [75], *Tep2,3* double mutant, *Tep2,3,4* triple mutant, and UAS-*Tep1* RNAi (ML2D) were described before [44]. Of note, the *Tep4* mutant is in a *yw* background; we have tested this mutant background in survival experiments and it did not differ significantly from the w^{A5001} background (Appendix Fig S6). The *Tep4* and the compound *Tep2,3,4* triple mutants are in distinct genetic backgrounds since the latter was generated by recombination between a *Tep4* mutant in a *yw* background and a *Tep2-3* chromosome in a w^{A5001} background [44]. Transgenic flies allowing to label hemocytes with GFP (hml::Gal4, UAS::GFP) were obtained by the Bloomington *Drosophila* Stock Center (BL70140). c564 *Gal* $Gal80^{ts}$ and hml *Gal4* $Gal80^{ts}$ fly lines were used as drivers for UAS-*Tep1* RNAi [76]. These crosses were done at 18°C. After hatching, F1 flies were transferred to 29°C for 6 days to allow a strong expression of the *Tep1* RNAi construct. To block the phagocytosis ability of *Drosophila*, latex beads were injected in these flies 1 day before infection or upon the infection (Fig 2) as described before [73], except that flies were allowed to recover for 4 h after injection prior to being exposed to the bacteria.

Experiments were performed on 3- to 10-day-old female flies devoid of known pathogens, including Nora virus and microsporidia. Sample size was chosen according to the type of experiments and experiments repeated as needed.

Bacteria strains and growth conditions

Construction of the PA14 *ΔrhlR* mutant was described in Limmer *et al* [25]. The PA14 *ΔrhlI* in-frame deletion mutant was generated by replacing 2.018 kb of wild-type PA14 sequence with a 1.502-kb PCR-amplified fragment that contained a 0.516-kb deletion in the *rhlI* ORF. The PCR-amplified fragment containing the deleted gene was subcloned into the XbaI site of pEX18Ap [77], generating

plasmid pEX18*rhlI*Δ9. The resulting construct was used to introduce the *rhlI*-deleted gene into the wild-type PA14 genome by homologous recombination, resulting in the *ΔrhlI* 9-9 mutant. Colonies of putative mutants obtained after homologous recombination were screened by PCR using appropriate flanking primers and were further confirmed by sequencing the corresponding PCR products.

P. aeruginosa strain PA14 wild-type [78], *ΔrhlR* (*rhlR*), *ΔrhlI* (*rhlI*), *ΔlasR,* and *ΔlasI* mutants [25,63] were grown in brain–heart infusion broth (BHB), overnight, at 37°C with agitation.

Drosophila intestinal infections

Infections were performed as described previously with PA14 [73]. At least 20 flies were assessed per infection tube. Infected and control flies were kept at 25°C. For survival assays, the number of surviving flies was computed daily.

C. elegans killing and intestinal accumulation assays

The "slow killing" of *C. elegans* by *P. aeruginosa* PA14 wild-type and PA14 *rhlR* and *rhlI* mutants was monitored using automated image analysis as previously described [64,79]. To monitor the accumulation of *P. aeruginosa* in the *C. elegans* intestine, wild-type (N2) animals were used for all experiments. Worms were reared on non-pathogenic *E. coli OP50* on nematode growth media at 25°C. Synchronized L4 worms (fourth larval stage) were transferred to slow kill (SK) nematode growth media agar plates containing a lawn of *P. aeruginosa* PA14::GFP. Post-infection at 24 and 48 h, approximately 20 worms were picked onto a 2% agar pad that contain the paralyzing agent levamisole (1 mM). The worms were imaged in the GFP channel using a Zeiss Apotome microscope with the same exposure time for all the worms on wild-type PA14 and the *rhlR* and *rhlI* mutants. The images were processed post-acquisition using ImageJ software, and the area and fluorescence intensity were measured. The relative fluorescence intensity is plotted.

Analysis of hemocyte recruitment at the gut upon intestinal infection with PA14 or mutant bacteria

Female transgenic flies *hml*::*Gal4*, UAS::GFP, 3–5 days old were kept at 29°C for 3 days to induce the expression of the GFP. Flies were infected with wild-type PA14, *rhlR,* or *rhlI* as described above. Non-infected flies (kept on sucrose only) were used as control. At determined time points, flies were dissected and the gut was removed for analysis. In order to maintain the attachment of hemocytes to gut, flies were directly dissected in 4% PFA and processed as described before [65]. Guts were analyzed using a ZEISS LSM700 confocal, and hemocytes attached to the gut were manually counted.

In vivo phagocytosis assay of pHrodo®-labeled heat-killed bacteria

The pHrodo® dye becomes fluorescent when placed in an acidic environment such as that encountered in phagosomes. Wild-type PA14 and *rhlR* overnight cultures in BHB at 37°C were washed in PBS and heat-killed at 65°C for 60 min. Killed bacteria were then washed twice in PBS and labeled with pHrodo® following the manufacturer's instructions (pHrodo® Red succinimidyl ester, #P36600, Molecular Probes). Solutions at 5×10^{10} bacteria/ml were further aliquoted and kept frozen at −20°C before use. A5001 or *Tep4* third-instar wandering larvae (n = 23–35) were injected with 27.6 nl of the pHrodo-labeled bacteria solutions, using a Nanoject apparatus (Drummond). After 40 min of incubation, one larva was bled in each well of an 8-well pattern microscopy slide that contained a 0.5 μg/ml DAPI solution in PBS. The cells were left to settle to the bottom of the well for 30 min in a humid chamber. The slides were mounted in PBS and immediately analyzed using a Zeiss Axioskope 2 fluorescent microscope. 10–80 cells per larva were analyzed: The number of red-fluorescent pHrodo-labeled bacteria was counted for each DAPI-positive hemocyte, and the phagocytosis index was calculated (percentage of phagocytes containing at least 1 bacterium) × (mean number of bacteria per phagocyte).

In vivo phagocytosis assay of live bacteria

Overnight cultures of wild-type PA14, *rhlR*, and *RhlI* were concentrated to OD_{600} = 20 in PBS. A5001 or *Tep4* third-instar wandering larvae (n = 20–38 for A5001; n = 4–8 for *Tep4*) were injected with 27.6 nl of the live bacteria solutions using a Nanoject apparatus (Drummond). After 40 min of incubation, one larva was bled in each well of an 8-well pattern microscopy slide that contained PBS. The cells were left to settle to the bottom of the well for 30 min and then were fixed in 1% paraformaldehyde for 10 min, in a humid chamber. The fixed cells were washed twice in PBS, and after a 30-min incubation in a PBS solution with 2% BSA, the samples were stained with a 1/100 to 1/500 diluted rabbit antiserum against PA14 in a PBS solution with 2% BSA overnight at 4°C or for 2 h at room temperature (the rabbit antiserum was raised against UV-killed wild-type PA14 by Covalab and also detects *rhlI* and *rhlR* bacteria). The cells were incubated with a Cy3-labeled goat anti-rabbit secondary antibody (Invitrogen) in a PBS solution with 2% BSA for 2 h at room temperature. After a 20-min permeabilization step in a PBS solution with 0.1% Triton X-100 and 2% BSA, a second round of staining with the diluted rabbit antiserum against PA14 in a PBS solution with 0.1% Triton X-100 and 2% BSA was performed overnight at 4°C. The samples were then incubated with a FITC-labeled goat anti-rabbit secondary antibody (Invitrogen) in a PBS solution with 0.1% Triton X-100 and 2% BSA for 2 h at room temperature. The slides were mounted in Vectashield with DAPI (Vector Laboratories) and analyzed using a Zeiss Axioskope 2 fluorescent microscope. 40–50 cells per larva were analyzed: The number of green fluorescent bacteria that were not also red (phagocytosed bacteria) was determined for each DAPI-positive hemocyte, and the phagocytosis index was calculated (percentage of phagocytes containing at least 1 only-green bacterium) × (mean number of only-green bacteria per phagocyte).

Opsonization assay of live bacteria

Overnight cultures of PA14 wild-type, *rhlR*, and *RhlI* mutants were concentrated to OD_{600} = 10 in PBS. Twenty third-instar larvae were bled in 150 μl of bacteria, resuspended in PBS at OD_{600} = 10, and incubated at room temperature for 30–45 min to allow a hypothetical Tep4-dependent opsonization to occur. Samples were centrifuged at

500 *g* for 15 min, and the pellet (containing larval debris) was removed. A second centrifugation was performed at 3,500 *g* for 15 min to retrieve bacteria in the pellet, which were resuspended in 10 µl PBS. These bacteria were then injected into either naive wild-type or *Tep4* mutant larvae prior to bleeding these injected larvae to be able to count the ingested bacteria as described above. In some experiments, a 1:1(v/v) solution from the PA14 wild-type and *rhlR* solutions was prepared. A5001 or *Tep4* third-instar larvae were injected with 32.2 nl of the live bacteria solutions using a Nanoject apparatus (Drummond). After 60–90 min of incubation, one larva was bled in each well of an 8-well pattern microscopy slide that contained PBS and stained as described in the previous section.

Phenoloxidase cleavage assay

The procedure was performed as described [80], except that hemolymph loads were not adjusted by measuring the protein content of the extracted hemolymph. The antibody was generated by Dr. H. M. Müller against *Anopheles* phenol oxidases [81]. The ratio of cleaved to non-cleaved form was determined by densitometry scanning.

Statistical analysis

Except when indicated, data were analyzed in a (generalized) linear modeling framework performed using R (version 3.4.2). Global differences in the mean response (e.g., LT50, bacterial counts or phagocytosis index) between the different experimental conditions (bacteria and fly genotypes, latex bead injected, and other experimental treatments) and their combinations were tested by analysis of variance/deviance (Anova function in the "car" package). Pairwise comparisons between specific conditions and "differences of differences" (e.g., comparison of the difference in LT50 between wild-type and RhlR bacteria in WT and phagocytosis-impaired flies) were done using the *lsmeans* and *contrast* function of the "lsmeans" package. Normally (Gaussian) distributed data (e.g., LT_{50}s, qPCR datasets) were tested using linear models (*lm* function in "base" R). Count data (*i.e.*, phagocytosis index and bacterial counts) were analyzed using generalized linear models (GLM) assuming a negative binomial distribution (*glm.nb* function in the "MASS" package). This distribution is an extension of the Poisson distribution (commonly used to model count data), which allows inequality between means and variances. Normality of the residuals was assessed visually by quantile plots and tested by the Shapiro–Wilk test. Reported *P*-values for tests involving multiple comparisons were adjusted using a sequential Bonferroni correction. Survival curve statistical analysis (Fig 7) was performed using the log-rank (Mantel–Cox) as implemented in Graphpad Prism version 5 (Graphpad software Inc., San Diego, CA). Details are included in the legend of each figure. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

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Author contributions
SH, AF, SL, and DF conceived the *Drosophila* experiments and analyzed the data. SH performed the majority of these experiments, with later work performed by AF and SL; SS performed the precursor experiments that led to this work. Work on the septic injury model was performed by JC, with some help from ZL. *ΔlasR*, *ΔlasI*, *ΔrhlR*, and *ΔrhlI* mutants were constructed by ED and SY, except when indicated otherwise. AH and FMA conceived the *C. elegans* experiments and analyzed the data, which were obtained by AH. NM performed the statistical analysis. SH, DF, and FMA wrote the manuscript, with inputs from other co-authors.

Conflict of interest
The authors declare that they have no conflict of interest.

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